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# Anti-platelet therapy: phosphodiesterase inhibitors

**Paolo Gresele, Stefania Momi & Emanuela Falcinelli**

*Department of Internal Medicine, Section of Internal and Cardiovascular Medicine, University of Perugia, Italy*

**Correspondence**
Dr Paolo Gresele MD, PhD, Department of Internal Medicine, Section of Internal and Cardiovascular Medicine, University of Perugia, Via E. dal Pozzo, 06126 Perugia, Italy.
Tel.: +39 75 578 3989
Fax: +39 75 571 6083
E-mail: grespa@unipg.it

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Inhibition of platelet aggregation can be achieved either by the blockade of membrane receptors or by interaction with intracellular signalling pathways. Cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP) are two critical intracellular second messengers provided with strong inhibitory activity on fundamental platelet functions. Phosphodiesterases (PDEs), by catalysing the hydrolysis of cAMP and cGMP, limit the intracellular levels of cyclic nucleotides, thus regulating platelet function. The inhibition of PDEs may therefore exert a strong platelet inhibitory effect. Platelets possess three PDE isoforms (PDE2, PDE3 and PDE5), with different selectivity for cAMP and cGMP. Several nonselective or isoenzyme-selective PDE inhibitors have been developed, and some of them have entered clinical use as antiplatelet agents. This review focuses on the effect of PDE2, PDE3 and PDE5 inhibitors on platelet function and on the evidence for an antithrombotic action of some of them, and in particular of dipyridamole and cilostazol.

## Introduction

Inhibition of platelet aggregation has shown great benefit for the treatment and prevention of ischaemic cardiovascular disease. Platelet inhibition can be achieved either by blockade of membrane receptors or by interaction with intracellular signalling pathways. While receptor antagonism may provide high specificity, the inhibition of platelet signal transduction may display broader effects, suppressing platelet activation regardless of the initial stimulus. Inhibition of signalling can be obtained either by interfering with platelet-activating second messengers or by amplifying the action of physiological platelet inhibitors, such as endothelium-derived prostacyclin ($PGI_2$) and nitric oxide (NO), which act by activating adenylyl and guanylyl cyclases respectively, thus increasing intraplatelet cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP). cAMP and cGMP are two critical inhibitory intracellular second messengers regulating fundamental platelet processes. In fact, elevation of platelet cyclic nucleotides interferes basically with all known platelet activatory signalling pathways and thus blocks cytoskeletal rearrangement, fibrinogen receptor activation, degranulation and expression of pro-inflammatory mediators. Indeed, cAMP and cGMP activate protein kinases that phosphorylate specific substrates (i.e. Rap1, MLCK, vasodilator-stimulated phosphoprotein, etc.) thus interfering with receptor/G-protein activation, phospholipase C, protein kinase C and mitogen-activated protein kinase activation, and blocking cytosolic $Ca^{2+}$ elevation and the reorganization of the cytoskeleton [1].

## Phosphodiesterases

Phosphodiesterases (PDEs), by catalysing the hydrolysis of cAMP and cGMP to inactive 5′-AMP and 5′-GMP, limit intracellular levels of cyclic nucleotides and thus regulate the amplitude, duration and compartmentation of cyclic nucleotide signalling [2].

To date, more than 60 different isoforms of PDE have been described in mammalian tissues, grouped into 11 broad families (PDE1–PDE11) based on differences in their structure, kinetics, regulatory properties and sensitivity to chemical inhibitors. Alternative splicing and transcription start sites also contribute to the multiplicity of the different isoforms, many of which possess species-specific tissue and/or cellular distribution. Current data suggest that individual isozymes modulate distinct regulatory pathways in cells.

Given that PDEs are associated with many physiological functions, targeting pathological conditions by

## Table 1

Phosphodiesterase (PDE) families, tissue expression, their inhibitors and their role in disease

| Family | Substrate | Tissue expression | Inhibitors | Disease targets |
|---|---|---|---|---|
| **PDE1** | cGMP > cAMP | Heart, vascular smooth muscle and brain | Vinpocetine, IC86340 | Cerebrovascular disorders and age-related memory impairment, cardiac hypertrophy |
| **PDE2** | cGMP = cAMP | **Platelets**, heart and endothelial cells | EHNA, EHNA analogues: BAY 60-7550, PDP | Memory impairment , endothelial permeability in inflammatory conditions |
| **PDE3** | cAMP > cGMP | **Platelets**, vascular smooth muscle, corpus cavernosum and heart | **Cilostazol, milrinone**, vesnarinone, lixazinone, anagrelide | Peripheral vascular disease, congestive heart failure, airways disease, fertility, ischaemic cardiovascular disease |
| **PDE4** | cAMP | Lung, heart, vascular smooth muscle, brain, inflammatory and immune cells | Rolipram, etazolate, zardaverine | Chronic obstructive pulmonary disease, asthma, allergic disease |
| **PDE5** | cGMP | **Platelets**, vascular smooth muscle and corpus cavernosum | **Sildenafil**, vardenafil, tadalafil, zaprinast, **dypiridamole** | Erectile dysfunction, ischaemic cardiovascular disease |
| **PDE6** | cGMP > cAMP | Retinal rods and cones | Sildenafil, zaprinast, dypiridamole | None |
| **PDE7** | cAMP > cGMP | T cell, B cell, skeletal muscle and heart | BRL 50481, IC242, dypiridamole | Inflammation, osteoporosis |
| **PDE8** | cAMP | Testis, eye, liver, kidney, skeletal muscle, embryo, ovary and brain | Zaprinast | None |
| **PDE9** | cGMP | Brain, small intestinal smooth muscle, liver, kidney, lung, testis, skeletal muscle and heart | BAY 73-6691 | Alzheimer's disease |
| **PDE10** | cAMP > cGMP | Testis and brain | None | None |
| **PDE11** | cAMP = cGMP | Skeletal muscle, prostate, kidney, liver, pituitary, salivary glands and testis | None | None |

In bold are the drugs discussed in detail in the manuscript. PDE, phosphodiesterase; cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate.

modulating individual PDEs has been the object of intense study (Table 1).

Functionally, phosphodiesterases can be classified in terms of their affinity and rates of degradation for cAMP and cGMP. The isoforms PDE1, 2 and 3 hydrolyse both cAMP and cGMP, whereas PDE4 and PDE8 specifically hydrolyse cAMP, and PDE5 specifically hydrolyses cGMP. Concerning affinity for cyclic nucleotides, PDE3 isoforms are high-affinity enzymes, with $K_D$ values <150 nM, while other families, such as PDE1 and PDE5, have affinities in the micromolar range. For many PDEs, affinity for one substrate is so much higher than for the other as to render them functionally monospecific. The isoform PDE3 has a relatively high affinity for both cAMP and cGMP, but a much lower efficacy of hydrolysis for cGMP, behaving essentially as a pure cAMP PDE; it has also been referred to as cGMP-inhibited cAMP PDE because cGMP may competitively inhibit AMP hydrolysis [3]. Thus, in platelets, agents that activate guanylyl cyclase potentiate the effects of activators of adenylyl cyclase [4].

Platelets express three PDE isoenzymes: PDE2, PDE3 and PDE5 (Table 2). Hidaka and Asano [5] were the first to resolve the PDE activity of platelets into three distinct peaks, as follows: the first prefers cGMP as a substrate, with a $K_m$ of about 1 μM, and is selectively inhibited by PDE5 inhibitors; the second hydrolyses cAMP and cGMP equally well [6] and is selectively inhibited by PDE2 inhibitors; and the third has a high affinity for both cAMP and cGMP, but hydrolyses cAMP much more rapidly than cGMP, and is selectively inhibited by PDE3 inhibitors. Thus, in platelets cAMP is hydrolysed by PDE3 and PDE2, and cGMP is hydrolysed by PDE5 and PDE2 (Figure 1); these isozymes account for at least 90% of platelet PDE activity.

## Phosphodiesterase inhibitors

Soon after the discovery of PDEs, it was found that caffeine is an effective inhibitor of PDE activity, and a number of nonselective PDE inhibitors, including the caffeine analogue theophylline, entered clinical use. Since then, several isoenzyme-selective PDE inhibitors have been developed as therapeutics or are currently under investigation for various disorders.

This review focuses on the effects of PDE2, PDE3 and PDE5 inhibitors on platelet function and on their potential as antithrombotic agents.

## Nonselective PDE inhibitors: methylxanthines and pentoxifylline

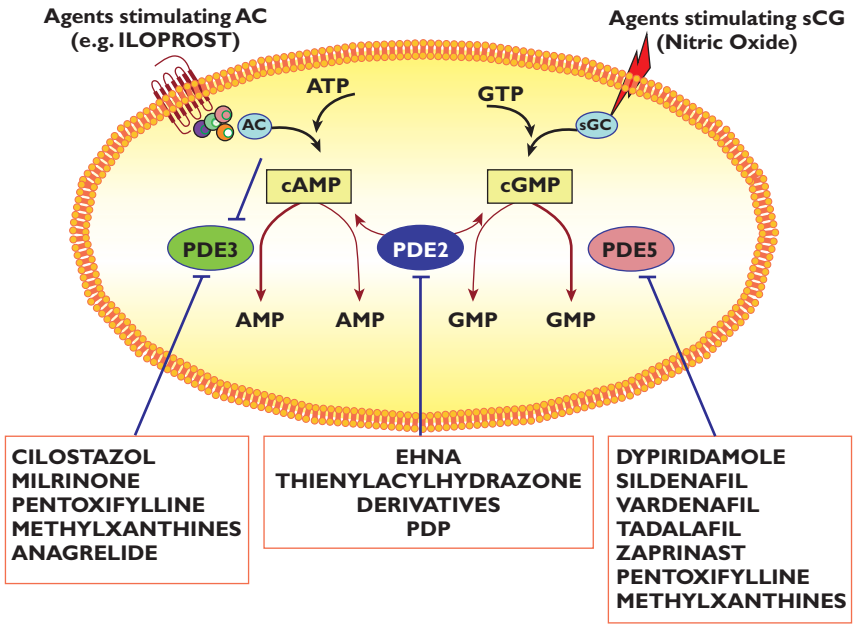
Naturally occurring methylxanthines were the earliest PDE inhibitors to be discovered, and among them, caffeine (1,3,7-trimethylxanthine) was the first [7]. Some years later, theophilline (1,3-dimethylxanthine) and theobromine, more effective PDE inhibitors, were characterized [8]. Ardlie and co-workers documented inhibition of platelet aggregation by caffeine and theophylline in 1967 [9]. In a milestone study in 1971, Mills and Smith showed that

## Table 2

Phosphodiesterases (PDEs) in platelets and their inhibitors

| Family | Substrate | Inhibitors | Phase of development | Indication |
|---|---|---|---|---|
| **PDE2** | cGMP = cAMP | EHNA | Preclinical | Endotoxaemia |
| | | BAY 60-7550 | Preclinical | Cognitive impairment |
| | | PDP | Preclinical | Cognitive impairment |
| **PDE3** | cAMP > cGMP | **Cilostazol** | Registered | Intermittent claudication; peripheral arterial disease |
| | | Milrinone | Registered | Congestive heart failure |
| | | Vesnarinone | Phase II | Sarcoma Kaposi; HIV infection |
| | | Lixazinone | Preclinical | Polycystic kidney disease |
| | | Anagrelide | Registered | Essential thrombocythaemia |
| **PDE5** | cGMP | **Dypiridamole** | Registered | In association with aspirin for second prevention of ischaemic cerebrovascular disease; as adjunct to coumarin anticoagulants in the prevention of thromboembolic complications in patients with prosthetic heart valves |
| | | Sildenafil | Registered | Erectile dysfunction |
| | | Vardenafil | Registered | Erectile dysfunction |
| | | Tadalafil | Registered | Pulmonary arterial hypertension; erectile dysfunction |
| | | Zaprinast | Preclinical | Allergic disease |

In bold are the drugs discussed in detail in the manuscript. PDE2, phosphodiesterase inhibitor-2; PDE3, phosphodiesterase inhibitor-3; PDE5, phosphodiesterase inhibitor-5; cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; PDP, -(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6-one.




## Figure 1

Schematic representation of a platelet and the mechanisms regulating intraplatelet levels of cyclic nucleotides (cAMP and cGMP); the three isoforms of phosphodiesterases so far described in platelets and their pharmacological inhibitors are described. In bold are the drugs discussed in detail. AC, adenylate cyclase; sCG, solubile guanylate cyclase; ATP, adenosine triphoasphate; GTP, guanosine triphosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; cGMP, cyclic guanosine 3′,5′-monophosphate; AMP, adenosine 3′,5′-monophosphate; GMP, guanosine 3′,5′-monophosphate; PDE2, phosphodiesterase inhibitor-2; PDE3, phosphodiesterase inhibitor-3; PDE5, phosphodiesterase inhibitor-5

adenosine increases cAMP in platelets and that methylxanthines prevent the conversion of cAMP to AMP, acting as PDE inhibitors, thus greatly increasing the inhibitory effects of adenosine on platelet aggregation [10]. Caffeine and theophylline act also as nonselective adenosine receptor antagonists [11]. In healthy volunteers, 250 mg caffeine orally three times a day for 7 days reduced platelet aggregation, increased intracellular cAMP and upregulated the platelet adenosine $A_{2A}$ receptors [12].

Pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-xanthine), a vasoactive drug used in patients with intermittent claudication [13] and reported to reduce whole blood viscosity

and to improve erythrocyte deformability, was shown to inhibit platelet aggregation *in vitro* in platelet-rich plasma, but at concentrations higher than those attainable *in vivo* [14]. However, given that the platelet inhibitory effect was potentiated by $PGI_2$, it was suggested that the *in vivo* antiplatelet activity could be stronger [15]; moreover, pentoxifylline was found to inhibit platelet aggregation in whole blood more effectively than in platelet-rich plasma, due to the contribution of an adenosine uptake-inhibitory effect on erythrocytes [16]. So far, there is no evidence that pentoxyfilline reduces ischaemic cardiovascular events [13].

## PDE2 inhibitors

The isoform PDE2 hydrolyses both cAMP and cGMP, and high concentrations of cGMP stimulate PDE2 [2].

Inhibitors of PDE2 have been used mainly as research tools, but ongoing studies investigate their effectiveness for memory impairment and prevention of endothelial permeability in inflammation [2].

Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), an inhibitor of adenosine deaminase (ADA), was shown to act as a selective PDE2 inhibitor with an $IC_{50}$ of 3 μM and at least 50-fold selectivity over other PDEs [17]. EHNA (20 μM) has no direct effect on platelet aggregation, but it potentiates the inhibition of thrombin-induced platelet aggregation by nitroprusside, a guanylyl cyclase stimulator [17]. The use of EHNA as a tool to assess the role of PDE2 in platelets is limited by the low PDE2-inhibitory potency and by the concomitant adenosine deaminase inhibitory action.

Recently, a new series of thienylacylhydrazone derivatives synthesized from natural safrole (4-allyl-1,2-methyldioxybenzene), a Brazilian natural product obtained from *Ocotea pretiosa*, have been studied as PDE2 inhibitors for their antiplatelet activity [18]. The most potent of these inhibited platelet aggregation induced by arachidonic acid ($IC_{50}$ 0.2–3.1 μM) and collagen ($IC_{50}$ 0.9–3.4 μM), effects enhanced by sodium nitroprusside without interference with ADP-induced aggregation, ATP release and thromboxane (Tx)B2 production [19]. Moreover, one of these induced a concentration-dependent relaxation of intact rat aortic rings ($IC_{50}$ 74 μM) [20].

Novel selective PDE2 inhibitors with nanomolar potency, such as 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6one (PDP), have recently been developed but not tested on platelets [21].

## PDE3 inhibitors

The isoform PDE3 comprises two subfamilies, PDE3A and PDE3B, showing distinct and overlapping tissue and subcellular distributions. *In situ* hybridization studies have shown that PDE3A is highly expressed in the cardiovascular system, including the myocardium, vascular smooth muscle cells and megakaryocytes, while PDE3B mRNA is detected in adipocytes [22]. Recently, it has been shown that PDE3A is the main subtype of PDE3 expressed in platelets [23].

### *Anagrelide*

Anagrelide (Agrylin/Xagrid; BL-4162A; 6,7-dichloro-1,5-dihydroimidazo[2, 1–6] quinazolin-2[3H]one monohydrochloride hydrate) is a potent inhibitor of PDE3 and a potent and broad-spectrum inhibitor of platelet aggregation ($IC_{50}$ = 36 nM) [24].

During studies in humans, however, anagrelide was found to produce thrombocytopoenia [25]; although the mechanisms through which anagrelide inhibits the megakaryocytes maturation are not completely understoood, the drug has entered clinical use for patients with essential thrombocythemia [26].

### *Cilostazol*

Cilostazol, a 2-oxo-quinoline derivative (6-(4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy)-3,4-dihydro-2(1H)-quinolinone), was registered in Japan and other Asiatic countries in 1988 and approved in the USA in 1999 for the treatment of intermittent claudication [27].

Cilostazol is a specific and strong inhibitor of PDE3 in platelets ($IC_{50}$ = 0.2 μM) and smooth muscle cells, where it diminishes intracellular calcium, causing smooth muscle cell relaxation and inhibition of platelet activation [28]. Cilostazol also inhibits adenosine uptake, thus enhancing adenosine levels that in turn enhance intracellular cAMP, resulting in additional increases in cAMP [28].

*Platelet inhibition* Cilostazol inhibits both primary and secondary platelet aggregation induced by collagen, ADP, arachidonic acid and adrenaline with $IC_{50}$ values ranging from 3.6 to 15.0 μM, depending on the agonist [29, 30]. It also suppresses the expression of P-selectin ($IC_{50}$ 25 μM) [31], TxB2 production, platelet factor 4 and platelet-derived growth factor release, and enhances the antiplatelet effects of $PGI_2$ [32]. Cilostazol inhibits shear stress-induced platelet activation *in vitro*, with an $IC_{50}$ of 15.0 μM [33], and *ex vivo* is not dissimilar from ticlopidine plus aspirin [34]. Among patients with acute myocardial infarction undergoing coronary stenting, the association of cilostazol with clopidogrel plus aspirin resulted in a greater antiplatelet effect in comparison to clopidogrel plus aspirin [35]. Moreover, the addition of cilostazol to clopidogrel gives greater inhibition of platelet aggregation than clopidogrel, either at the standard or at double dose, in clopidogrel low responders [36].

One potential benefit of the use of cilostazol over conventional antiplatelet therapy is the relatively short recovery time of platelet function [37].

Cilostazol inhibits the expression of monocyte chemoattractant protein-1, an initial trigger in the

development of atherosclerosis, in human umbilical vein endothelial cells by increasing intracellular cAMP [38].

Cilostazol is rapidly absorbed, and reaches peak plasma concentrations (775 ng $ml^{-1}$ = 2.09 μM) at about 2.4 h after oral administration. In plasma it is largely bound to proteins (95–98%), primarily albumin [39, 40]. Specifically, metabolism of cilostazol occurs primarily via CYP3A5 and, to a lesser extent, CYP2C19, while <1% of the administered dose is excreted unchanged in urine [41]. The CYP3A5 and CYP2C19 polymorphisms explain the substantial interindividual variability in the metabolism of cilostazol, with a coefficient of variation of about 40–60% [39, 42].

After oral administration, cilostazol and its metabolites, 3,4-dehydrocilostazol (OPC-13015) and 4-*trans*-hydroxycilostazol (OPC-13213), show a half-life of approximately 10 h, with a twofold accumulation during repeated administration [28].

*Effect on bleeding time* There is no evidence that cilostazol prolongs the bleeding time when compared with aspirin, clopidogrel or ticlopidine [43, 44], even when used in combination with these antiplatelet drugs [44].

In a study in healthy men comparing ticlopidine, aspirin and cilostazol, bleeding time was significantly prolonged by aspirin and ticlopidine but not by cilostazol [45].

In patients with peripheral arterial disease, aspirin or clopidogrel alone significantly prolonged bleeding time, and even more when used in combination. In contrast, no prolongation of the bleeding time was observed with cilostazol alone, nor was there a further prolongation when added to aspirin or clopidogrel [46].

*Animal models* Cilostazol exerts antithrombotic activity in different animal models. Pulmonary thromboembolism induced by ADP and collagen in mice was reduced by cilostazol at 3 and 10 mg $kg^{-1}$ *per os*, more effectively than by aspirin and pentoxifylline [29]. Cerebral infarction induced by the injection of arachidonic acid into the internal carotid artery of rabbits was reduced by 1 mg $kg^{-1}$ cilostazol [47].

In a porcine model of totally occlusive thrombosis of the carotid artery induced by electrical injury, cilostazol significantly prolonged the time to occlusion and decreased thrombus weight more than ticlopidine [48]. In $FeCl_3$ venous thrombosis model in rats, oral administration of 50 mg $kg^{-1}$ cilostazol significantly decreased venous thrombus weight [49].

*Clinical studies* Several studies have shown the superiority of cilostazol *vs* placebo and also *vs* pentoxifylline in increasing the walking distance [50] and quality of life of peripheral arterial disease patients [51].

Cilostazol was tested for secondary prevention of stroke in a randomized, placebo-controlled, double-blind trial in 1052 patients in Japan [52]. Cilostazol significantly reduced the recurrence of ischaemic stroke (–41.7%; confidence interval (CI) –9.2–62.5%) compared with placebo, and the incidence of the combined end-point of myocardial infarction, transient ischaemic attack and intracranial haemorrhage. The benefits were achieved without a significant increase of bleeding (2.8 *vs* 2.1%) and with no fatal intracranial haemorrhage [52]. These data seem to confirm a low bleeding risk with cilostazol. In the second Cilostazol Stroke Prevention Study (CSPS 2), in 2757 patients with a noncardioembolic cerebral infarction within the previous 26 weeks, cilostazol 100 mg twice daily resulted to be noninferior, and possibly superior, to aspirin 81 mg once daily, in preventing stroke recurrence (yearly rate of 2·76% ($n$ = 82) in the cilostazol group and 3·71% ($n$ = 119) in the aspirin group, hazard ratio 0·743, 95% CI 0·564–0·981; $P$ = 0·0357) and was associated with fewer haemorrhagic events [53]. A recent meta-analysis of 12 double-bind, placebo-controlled trials in patients with atherothrombotic disease concluded that cilostazol is associated with a significant risk reduction of cerebrovascular events (relative risk [RR] 0.86; 95% CI 0.74–0.99; $P$ = 0.038), with no associated increase of bleeding [54]. There was no significant difference in the incidence of cardiac events (RR 0.99; 95% CI 0.83–1.17; $P$ = 0.908) [54].

In a study in 1212 patients with acute coronary syndromes undergoing percutaneous coronary intervention, cilostazol added to aspirin plus clopidogrel reduced the composite end-point of cardiac death, myocardial infarction and stroke, in comparison to aspirin plus clopidogrel [55].

Recently, however, a multicentre randomized trial in 960 patients undergoing coronary drug-eluting stent implantation showed that the addition of cilostazol to aspirin and clopidogrel for 6 months did not reduce the composite adverse outcome of cardiac death, nonfatal myocardial infarction, ischaemic stroke or target lesion revascularization [56].

Cilostazol has also been suggested to prevent poststent restenosis [57]. Cilostazol was compared with ticlopidine, both in addition to aspirin, in 397 patients undergoing elective coronary stenting for the rate of restenosis; restenosis tended to be lower with cilostazol, but not significantly [58].

A recent study investigated whether periprocedural cilostazol affects the incidence of in-stent restenosis (ISR) or target vessel revascularization (TVR) after carotid artery stenting. The study group comprised 553 patients undergoing carotid artery stenting followed for 30 months, 207 of whom (37.4%) were treated with cilostazol. The incidence of ISR or TVR was significantly lower in the cilostazol-treated group than in the untreated group (1.4 *vs* 6.4%; log-rank $P$ = 0.006) [59]. A metanalysis of five randomized controlled trials comparing triple therapy cilostazol plus aspirin plus thienopyridines with aspirin plus clopidogrel, involving 1597 patients undergoing coronary stenting, showed that triple therapy significantly reduced the 6 months restenosis rate (12.7 *vs* 21.9%; odds ratio 0.5; 95% CI 0.38–0.66; $P$ < 0.001) [60].

Finally, in a small study, cilostazol administration improved long-term patency after carotid artery stenting in 97 patients with high-grade carotid stenosis monitored for a 12 month period (0 *vs* 15.7%, $P = 0.03$) [61].

The most common adverse effects of cilostazol are headache, tachycardia, palpitations, soft stools and diarrhoea [62].

### *Milrinone*

Milrinone, a specific PDE3A inhibitor, inhibits arachidonic acid-induced platelet shape change and platelet aggregation induced by ADP, U46619, collagen and calcium ionophore, both in whole blood and in platelet-rich plasma [63], with $IC_{50}$ values as low as 1 μmol $l^{-1}$, a concentration lower than the concentration achievable after therapeutic administration (1.5 μmol $l^{-1}$), indicating that an antiaggregating effect of milrinone can occur *in vivo* [64]. Milrinone induces an elevation of intraplatelet cAMP in a dose-dependent manner, resulting in the inhibition of platelet aggregation [65].

Milrinone is currently in clinical use for congestive heart failure [66].

## PDE3–PDE5 inhibitors

### *Dipyridamole*

Dipyridamole (2,6-bis (diethanolamino)-4,8-dipiperidino-pyrimido 5,4-d pyrimidine) was synthesized more than 50 years ago and initially used as a coronary vasodilator. However, it was soon shown that dipyridamole inhibited platelet aggregation [67, 68] and thrombus formation in rabbits [69], opening the way to the use of dipyridamole as an antithrombotic agent [70].

*Mechanisms of action* Dipyridamole affects platelet function by acting on the following different targets: it inhibits the reuptake of adenosine by red blood cells, in this way enhancing plasma levels of this vasodilator and platelet inhibitory nucleoside; it acts as an inhibitor of PDE5 and PDE3, thus increasing intraplatelet cAMP and/or cGMP; and it acts as a antioxidant by scavenging free radicals that inactivate cyclo-oxygenase, thus enhancing $PGI_2$ biosynthesis.

Adenosine is released by tissues in the extracellular space as a breakdown product of ATP during ischaemia, or by erythrocytes stressed by elevated shear forces, and is then taken up avidly by erythrocytes to keep plasma levels low. Inhibition of adenosine reuptake by dipyridamole is concentration dependent and reaches 90% at 1 μM of dipyridamole in whole blood [71], a concentration in the range of those attained after oral administration to humans (0.5–6 μM). Dipyridamole inhibits platelet aggregation in whole blood, as assessed by impedance aggregometry, but not in platelet-rich plasma both *in vitro* and *ex vivo*, by blocking the reuptake of adenosine [72, 73].

A stronger antiplatelet effect of dipyridamole in whole blood than in plasma was confirmed using different laboratory methods [74–77].

The slight PDE3-inhibitory action of dipyridamole increases the effects of adenosine and $PGI_2$, both stimulators of adenylylcyclase, leading to inhibition of platelet activation [78, 79].

Dipyridamole potentiates the inhibitory effects of NO on human and rabbit platelets also [80] by inhibiting PDE5 and thus increasing production of vasodilator-stimulated phosphoprotein, an established marker of the NO/cGMP effects [81], both *in vitro* and *in vivo* [82]. However, the inhibition of PDE5 is detectable *in vitro* only at concentrations (100–200 μM) much higher than those attainable after oral administration.

The antioxidant properties of dipyridamole may contribute to its antithrombotic effect. In fact, not only does dipyridamole enhance extracellular adenosine, which reduces superoxide anion generation by human neutrophils and directly scavenges oxygen- and hydroxyl-radicals, but it also has direct antioxidant effects. In fact, dipyridamole has better antioxidant properties than ascorbic acid, α-tocopherol and probucol [83, 84].

The antioxidant activity of dipyridamole leads to the inhibition of leukotriene B4 production *in vitro* by stimulated white blood cells [85]. Moreover, based on its antioxidant properties, dipyridamole was shown to prolong prostacyclin production by 'exhausted' vessel walls, preventing the autoinactivation of cyclo-oxygenase caused by enhanced peroxide formation [86], an activity similar to the so-called physiological prostacyclin-regulating plasma factor [87].

The redox state of dypiridamole regulates its antioxidant properties, which appear to be mediated in vascular cells by suppression of nuclear factor κB signalling [88].

Additional pharmacological effects of dipyridamole that may be of relevance for prevention of atherothrombosis are the inhibition of vascular smooth muscle cell proliferation, the prevention of endothelium–leukocyte interactions [89], the increase of antithrombotic properties of endothelial cells [90], the interference with interaction of leukocytes with endothelium, the enhancement of interleukin-1-stimulated NO production by smooth muscle cells [91], and the inhibition of inflammatory gene expression in platelet–monocyte aggregates [92]. Very recently, an additional interesting observation has shown that inhibition of multidrug resistance protein-4-mediated transport by dipyridamole increases aspirin entrapment in platelets and its *in vitro* effect on cyclo-oxygenase-1 activity [93], thus explaining in part the pharmacodynamic [94] and therapeutic [95, 96] synergism with aspirin.

*Antithrombotic properties* The antithrombotic effects of dipyridamole were evaluated in several animal models. Thrombus formation on air-injured carotid arteries of

rabbits was reduced by dipyridamole, while salicylate increased it [97]. In rabbits, the accumulation of radioactive fibrinogen at balloon angioplasty-treated carotid arteries was significantly reduced by dipyridamole (0.45 mg kg $h^{-1}$ for 4 h) in comparison to heparin [98].

More recently, in a model of chronic hindlimb ischaemia induced by the ligation of the common femoral artery branches in mice, dipyridamole (200 mg $kg^{-1}$ twice daily, for 7 days) was shown to enhance ischaemia-induced arteriogenesis, thereby quickly restoring blood flow through a protein kinase A-dependent NO pathway [99].

*Clinical studies* There is little clinical evidence that dipyridamole alone exerts an antithrombotic effect. Several studies instead have been carried out in combination with aspirin. Dipyridamole in combination with low-dose aspirin is associated with greater stroke risk reduction in patients with ischaemic cerebrovascular disease. This was confirmed in two large clinical studies: the ESPS2 [95], in which 6602 patients with prior stroke or transient ischaemic attack were randomized to aspirin (50 mg daily), slow release dipyridamole (200 mg twice a day), the two agents in a combined formulation, or placebo; and the ESPRIT trial [96], in which 2739 patients with previous transient ischaemic attack were randomized to aspirin (30–325 mg day) with or without dipyridamole (200 mg twice daily). A recent meta-analysis supports the higher efficacy of combination therapy over aspirin alone [100]. Based on these findings, the 2008 American College of Chest Physicians (ACCP) guidelines recommend dual therapy with extended-release dipyridamole plus aspirin over aspirin monotherapy for stroke prevention after a first transient ischaemic attack or stroke [101]. More recently, a large clinical trial, the PRoFESS study, carried out to investigate whether aspirin 25 mg and extended-release dypiridamole 200 mg twice a day was not inferior to clopidogrel 75 mg once a day in 20 332 patients with previous stroke, did not meet predefined criteria for non-inferiority, but showed similar rates of recurrent stroke with the two treatments.

There were more haemorrhagic strokes with aspirin plus extended-release dipyridamole than with clopidogrel [102]. The degree of functional impairment (disability and cognitive decline) at 3 months after stroke was similar across treatment arms [103].

Another condition for which there is evidence of efficacy of dipyridamole is secondary prophylaxis of thromboembolic events in patients with mechanical heart valves in combination with warfarin [104]. Concerning the effects in other clinical indications, a recent overview of 27 randomized long-term secondary prevention trials involving 23 019 patients presenting with an arterial vascular disease (cardiac disease, cerebral ischaemia, thrombosis of haemodialysis fistula, patients prone to develop atherosclerosis) comparing dipyridamole, alone or in combination with an antiplatelet drug other than dipyridamole (chiefly aspirin), with placebo found that there was no evidence that dipyridamole reduced the risk of vascular death, while it significantly reduced the risk of further vascular events (RR 0.88; 95% CI 0.81–0.95); however, this benefit was statistically significant only in patients presenting after cerebral ischaemia [105].

## PDE5 inhibitors

To date, only one family of PDE5, PDE5A, has been described. Three splice variants of PDE5A, PDE5A1/2/3, have been found in humans, differing in their N-terminal region, but with similar $K_m$ for cGMP [5].

Three PDE5 inhibitors, sildenafil (Viagra), vardenafil (Levitra) and tadalafil (Cialis), are currently in clinical use for erectile dysfunction.

Actually, the first developed PDE5 inhibitor to be used in humans was zaprinast (M&B22948), originally designed as a mast cell stabilizing drug for the treatment of allergic diseases [106]. The observation that cGMP elevation induced by zaprinast was associated with smooth muscle cell relaxation opened the possibility to use PDE5 inhibitors in cardiovascular diseases [107]. Zaprinast inhibits human platelet PDE5 with an $IC_{50}$ of 0.3 μM and PDE2A with an $IC_{50}$ of 42 μM. In association with sodium nitroprusside (1 μM), zaprinast led to a complete concentration-dependent inhibition of serotonin release in washed platelets ($IC_{50}$ ~1.6 μM) [108]. This compound was an unsuccessful clinical drug candidate, turning out to be weak and poorly selective for PDE5. Consequently, a variety of compounds with key variations in the heterocyclic ring system of zaprinast were prepared, leading to the identification in 1989 of sildenafil as a PDE5 inhibitor, which was 100 times as potent as zaprinast and highly specific in its action.

Sildenafil (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo [4,3-d]pyrimidin-5-yl) phenylsulphonyl]-4-methylpiperazine) is a potent PDE5 inhibitor, with high selectivity for human PDE5 over PDE2, PDE3 and PDE4 (>1000-fold), and moderate selectivity (>80-fold) over PDE1 [109]. Sildenafil instead is only ~10-fold more potent for PDE5 than for PDE6 (an enzyme found in the photoreceptors of the human retina), and this explains the frequent adverse effect of flashes of blue light with its use. Platelet PDE5 is inhibited by sildenafil with a $IC_{50}$ of 6.3 nM [110]. While sildenafil alone had no effect on platelet function, it potentiated the inhibition by sodium nitroprusside of rabbit and human platelets [110].

Sildenafil is rapidly absorbed after oral administration, with ~40% bioavailability. Plasma concentrations peak within 30–120 min (median, 60 min), and the drug is primarily metabolized by hepatic cytochrome P450 3A4 (major route) and 2C9 (minor route), which convert it to an active *N*-desmethyl metabolite that possesses 50% of the parent drug's potency for PDE5 [111].

The administration of sildenafil (100 and 50 mg in healthy volunteers) inhibited collagen-induced aggregation (100 mg), with an additive effect when combined with nitrates (isosorbide di-nitrate, 10 mg) [112]. Moreover, ADP-stimulated glycoprotein IIb/IIIa receptor activation was inhibited by sildenafil (100 mg) [113]. A study in healthy men showed that bleeding time was significantly prolonged (+72%) 1 h after sildenafil intake (100 mg), with recovery after 4 h, whereas a lower dose (50 mg) did not alter the bleeding time [112]. Sildenafil was also reported to increase the incidence of epistaxis in patients on concomitant vitamin K antagonists and in combination with heparin had an additive effect on bleeding time in rabbits [114]. Given the relatively low selectivity of sildenafil for PDE5, other more selective PDE5 inhibitors have been developed: vardenafil ($K_D$ of 0.6–0.7 nM; selectivity over PDE6, approximately 16-fold) [115] and tadalafil ($K_D$ range of 0.9–6.7 nM; 200–700 times more selective for PDE5 than PDE6) [116]. In patients with erectile dysfunction associated with cardiovascular risk factors (dyslipidaemia, hypertension and smoking), tadalafil enhanced cGMP and significantly reduced P-selectin expression in platelets [117].

## Conclusions

Antiplatelet therapy is the mainstay of treatment of patients with acute cardiovascular ischaemic events and of secondary prophylaxis of patients with a previous ischaemic event. Despite great progress, even optimal antithrombotic therapy still does not offer satisfactory protection against cardiovascular events and is associated with serious risk of haemorrhagic adverse effects. Agents targeting thromboxane production or activity, ADP and fibrinogen binding to platelets have proved of benefit but have also shown limitations.

An alternative, appealing strategy is to develop agents interfering with intracellular signalling pathways [118]. Phosphodiesterase inhibitors, by increasing crucial intraplatelet second messengers, such as cGMP and cAMP, have theoretically great potential for platelet inhibition. In fact, differently from aspirin or clopidogrel, not only they may inhibit platelet activation induced by whatever stimulus, but they may exert beneficial cardiovascular effects though their capacity to regulate the interaction of platelets with vascular cells in the setting of ischaemic cardiovascular disease. However, the widespread distribution of PDE in the body renders it difficult for an effective antiplatelet action be achieved without significant unwanted effects. Moreover, the reversibility of the effect of most clinically available PDE inhibitors on their target may represent a serious limitation for their antithrombotic effectiveness in long-term secondary prophylaxis. It is now established that continuous platelet inhibition is required in order to obtain an effective antithrombotic protection, and the reversibility of the blockade of the target, with temporary restoration of platelet function, in patients at high cardiovascular risk may be associated with enhanced ischaemic events, as shown by the negative experience with orally active glycoprotein IIb/IIIa antagonists [119]. Is not a chance that the antiplatelet agents currently used in long-term secondary prevention of ischaemic events, i.e. aspirin and thyenopiridines, both act by irreversibly blocking their target. A deeper understanding of the physiology of PDEs in platelets and other tissues, the development of techniques allowing the targeting of PDE inhibition to platelets and the development of irreversible or long-acting isoenzyme-selective PDE inhibitors may potentially lead to advances in antiplatelet therapy.

## Competing Interests

There are no competing interests to declare.

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